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Structural and functional properties of plasma membranes from the filamentous fungus *Penicillium chrysogenum*

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Functional plasma membranes from the filamentous fungus *Penicillium chrysogenum* have been isolated with the objective of studying transport processes. The isolation procedure consists of three steps, namely homogenization of cells with a Braun MSK homogenizer, followed by Percoll gradient centrifugation and floatation of membranes in a three-step Nycodenz gradient. This method can be applied to strains which differ significantly in morphology and penicillin-production capacity. Plasma membranes were fused with liposomes containing the beef heart mitochondrial cytochrome-*c* oxidase. In the presence of reduced cytochrome *c*, the hybrid membranes maintained a high proton motive force that functions as a driving force for the uptake of the amino acids arginine and valine via distinct transport systems.

For more than four decades penicillins have been commercially produced using the filamentous fungus *Penicillium chrysogenum*. This has prompted intensive research on the biochemistry and metabolic regulation of penicillin biosynthesis [1, 2]. Less information is available on the transport processes of precursors needed for the production of penicillin. The biosynthesis of penicillin proceeds in at least three cellular compartments, the cytosol, the vacuole and a microbody [3, 4]. This implies that precursors, intermediates and endproducts of penicillin synthesis have to cross several membranes. One or more of these transport steps may become limiting during the production of penicillin by the industrial strains that are presently used. Studies with intact mycelia demonstrate that *P. chrysogenum* contains transport systems that may play a crucial role in the biosynthesis of penicillin [5, 6].

Whole cells and, in particular, intact mycelia, are inadequate for transport studies as metabolism and compartmentalization of the transported compounds may interfere with a reliable interpretation of the results. Also, the morphology of mycelia differs for various strains. These problems can be avoided by the use of plasma membrane vesicles that are devoid of metabolic activities. Plasma membranes are routinely isolated from a wide variety of organisms and cell types. Filamentous fungi are a remarkable exception to this rule since only for a few species have isolation procedures been described [8–10]. For these few procedures mainly the

preparation of inside-out vesicles has been described, while in the case of right-side out vesicles no convenient method is available to energize the membrane. Although the *P. chrysogenum* plasma membranes contain a P-type H⁺-translocating ATPase, ATP cannot be used to energize right-side out membrane vesicles since when added from the outside ATP will not reach the catalytic side. Alternatively, artificially imposed ion-gradients can be used, but the application of this technique is limited as gradients rapidly decay. To study proton-motive force (Δp)-dependent transport systems, plasma membranes may be fused with liposomes containing an accessible primary proton pump. This approach has successfully been applied to both bacterial and yeast plasma membranes [7]. After fusion, the hybrid membranes are usually endowed with a low ion-permeability, and are thus able to sustain a high Δp for a considerable period of time.

In this study we present an isolation procedure that yields closed, transport-competent plasma membrane vesicles from the filamentous fungus *P. chrysogenum*. This procedure can be used for different strains. By fusing the plasma membranes with liposomes containing the beef heart mitochondrial cytochrome-*c* oxidase, a hybrid system is obtained that is active for the Δp -dependent uptake of amino acids. This study therefore demonstrates amino acid transport in plasma membranes derived from a filamentous fungus.

EXPERIMENTAL PROCEDURES

Organism and culture conditions

P. chrysogenum strains Wisconsin 54-1255 and Panlabs P2 (kindly supplied by Gist-brocades NV) were grown on production medium (pH 6.3) as described by Lara et al. [11] supplemented with 10 mM glutamate and 10% (mass/vol.) glucose. Cultures were incubated for approximately 70 h in a rotary shaker at 200 rpm and 25°C. Wisconsin strain 54-1255 was previously cultured for 24 h on production medium with the omission of phenylacetic acid and lactose, contain-

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Abbreviations. Δp , proton motive force; PhMeSO₂F, phenylmethylsulfonyl fluoride; CF₃OPh₂C(CN)₂, carbamoylcyanide-*m*-chlorophenylhydrazone; Ph(NMe₂)₂, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; $\Delta\psi$, transmembrane electrical potential; ΔpH , transmembrane pH gradient.

Enzymes. Cytochrome-*c* oxidase (EC 1.9.3.1); glucose-6-phosphatase (EC 3.1.3.9); α -D-mannosidase (EC 3.2.1.24); vanadate-sensitive H⁺-ATPase (EC 3.6.1.35).

ing 16% (mass/vol.) glucose. The P2 strain was previously cultured on YPG medium [1% (mass/vol.) yeast extract, 2% (mass/vol.) peptone and 2% (mass/vol.) glucose] for 72 h at pH 7.

Plasma membrane isolation

Mycelia were harvested by filtration and washed with an equal volume of 0.9% (mass/vol.) NaCl. All subsequent steps were performed at 4°C. Cells were suspended in cold 25 mM Mops/KOH, pH 7.2, 0.25 M sucrose, 5% (mass/vol.) glycerol, 1 mM MgCl₂, 2 mM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PhMeSO₂F), 0.15 mM tetracaine, 1 µg/ml leupeptin and 1 µg/ml pepstatin (buffer A), at 12.5 mg/ml and 7.5 mg/ml (dry masses) in the case of the P2 and Wisconsin 54-1255 strains, respectively. A 75-ml glass flask containing 50 g (P2) or 40 g (Wisconsin 54-1255) glass beads (1-mm diameter) was completely filled with suspended cells, and subsequently homogenized using a Braun MSK homogenizer for 2 min at full speed with cooling by liquid carbon dioxide expansion. Whole cells and debris were removed by centrifugation (3000×g for 5 min in a Beckman CS-6R swing-out rotor; the supernatant is referred to as the homogenate). Percoll was added to the supernatant (24%, final concentration) and the mixture was centrifuged for 30 min at 30000×g in a 45 Ti rotor. The upper band, which contained sealed plasma membranes, was removed and diluted fourfold with buffer A (the remainder of the Percoll gradient is referred to as fraction 1). Percoll was removed by centrifugation for 2 h at 100000×g in a 45 Ti rotor. Membrane pellets (on top of the Percoll pellet) were collected and suspended in buffer A (the final volume was 5% the volume of homogenate used; the supernatant is referred to as fraction 2). The membrane suspension (4.8 ml) was mixed with a stock solution of 50% (mass/vol. in buffer A) Nycodenz (Sigma; 3.2 ml), and poured into a thick-walled tube. Subsequently, 6 ml 15% (mass/vol.) Nycodenz (in buffer A) and 4 ml buffer A were added on top of this mixture. The gradient was centrifuged in a SW 28 rotor for 1.5 h at 90000×g. Sealed plasma membranes were recovered as a white band at the interphase of 15% Nycodenz and buffer A. Interphases were collected and diluted tenfold with buffer A (the remainder of the Nycodenz gradient is referred to as fraction 3). The diluted membrane suspension was centrifuged for 30 min at 25000×g in a SS34 rotor, and the plasma membranes, recovered as a pellet, were suspended in a small volume of buffer A and were stored under liquid N₂.

Marker enzyme activities

Vanadate-inhibited ATPase activity was determined as described by Widell et al. [12] at pH 6.3. Triton X-100 was added to a final concentration of 0.05% (mass/vol.). ATP hydrolysis was measured by the release of inorganic phosphate with malachite green [13] in the presence of 0.1% (mass/vol.) Triton X-100. Nitrate-sensitive ATPase activity (inhibition by 25 mM nitrate) was determined at pH 7.5 in the same way as vanadate-inhibited ATPase activity. Cytochrome-c oxidase was determined at pH 7.5 as described by Storrie et al. [14], and glucose-6-phosphatase was determined in the presence of 0.1% (mass/vol.) Lubrol PX using the coupled assay described by Gierow et al. [16]. Measurements were performed with an Aminco DW2000 spectrophotometer. α-D-Mannosidase was determined at pH 5.5 in the presence of 0.1% (mass/vol.) Lubrol PX, using a fluorometric

assay described by Faber et al. [15]. Measurements were performed on a Perkin-Elmer LS50B luminescence spectrometer. All marker enzymes were assayed at 25°C.

Electron microscopy studies

Phosphotungstic acid was used at low pH for cytochemical staining of plasma membranes as described by Roland et al. [17]. Glutaraldehyde/osmium tetroxide fixed, Epon-embedded membranes were, after etching, stained in the presence of chromic acid. The ultrathin sections were examined in a Philips CM-10 electron microscope. To quantify the amount of stained membranes a simple morphometric procedure was used [18]. A transparent overlay bearing parallel lines 1 cm apart was placed over electron micrographs at a final magnification of approximately 35000-fold. Intercepts of lines with membranes were counted and the amount of stained membranes was calculated as intercepts with stained membranes/100 total intercepts. Freeze fracture electron micrographs were prepared from replicas of membranes frozen in liquid nitrogen (N₂ slush). The replicas were examined with the same electron microscope as the ultrathin sections.

Orientation of membrane vesicles

The orientation of membrane vesicles was determined by trypsin inhibition of ATPase activity [19]. Membranes were digested with trypsin in the absence or presence of 0.1% (mass/vol.) Triton X-100 for 10 min at 25°C. Trypsin inhibitor was added, and the ATPase activity of the samples was determined in the presence of 0.1% (mass/vol.) Triton X-100.

Membrane fusion

Bovine heart mitochondria were obtained according to the procedure described by King [20]. Cytochrome-c oxidase was isolated from these mitochondria as described by Yu et al. [21], suspended in 50 mM sodium phosphate (pH 7.5) containing 1.5% (mass/vol.) cholic acid and stored in liquid nitrogen. Cytochrome-c oxidase was reconstituted in liposomes, composed of 75% (by mass) acetone/ether-washed *Escherichia coli* lipid and 25% (by mass) egg yolk L-phosphatidylcholine, at a ratio of 0.16 nmol heme a/mg lipid [22]. Cytochrome-c-oxidase-containing liposomes (10 mg lipid) and plasma membranes (1 mg protein) were mixed, rapidly frozen in liquid N₂ and thawed slowly at 21°C [7]. The freeze-thaw step was repeated once, and hybrid membranes were sized with a small-volume extrusion apparatus (Avestin Inc., Ottawa, Canada) [23] using polycarbonate filters (Avestin) with pore sizes of 400 nm and 200 nm diameter. Fused membranes had a protein/lipid ratio of approximately 0.08–0.09 (mass/mass) (relative to phospholipid).

Transport studies

Uptake of the amino acids arginine and valine was studied at 25°C. Cells were suspended in 50 mM potassium phosphate (pH 6.5) at final densities of 10 mg/ml (P2) or 6 mg/ml (Wisconsin 54-1255; dry masses). L-[U-¹⁴C]arginine (Amersham, 38 Ci/mol) or L-[U-¹⁴C]valine (Amersham, 28 Ci/mol) were added to the cell suspension to 30 µM. At given time intervals, samples of 0.5 ml were taken, added to 2 ml ice cold 0.1 M LiCl, and immediately filtered on 0.45-µm pore-size diameter cellulose-nitrate filters (Schleicher &

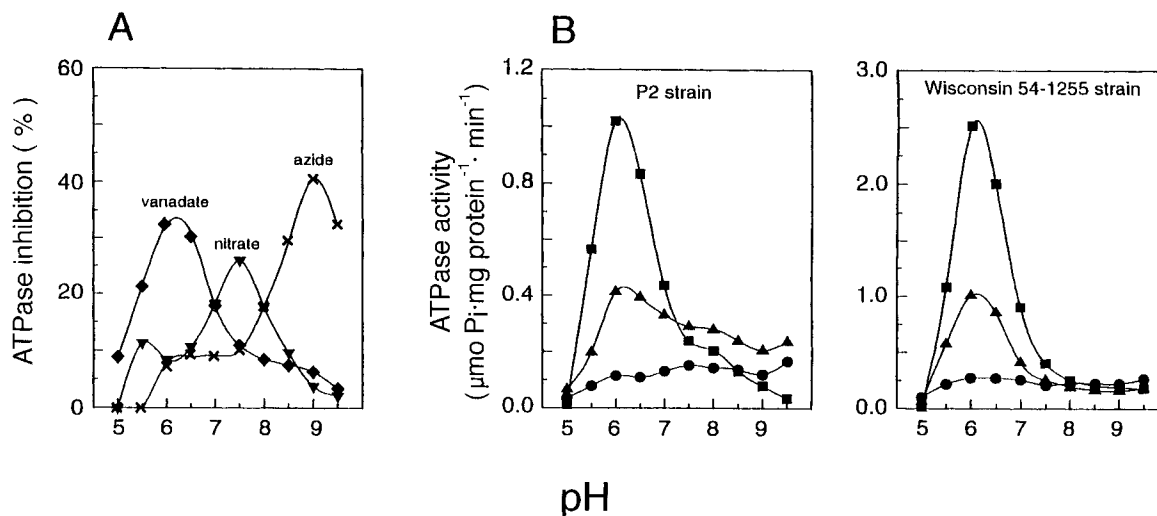


Fig. 1. ATPase activity of cellular fractions. (A) Inhibition of different ATPases by specific inhibitors. Inhibition of ATPase activity in the homogenate of the P2 strain at different pH by 100 μ M vanadate (\blacklozenge), 25 mM KNO_3 (\blacktriangledown) and 2 mM azide (\times). (B) pH dependency of ATPase activity in different membrane fractions. The ATPase activity in the homogenate (\bullet) of the P2 strain (left panel) or the Wisconsin 54-1255 strain (right panel) is shown for both a partial purified membrane fraction obtained after the Percoll gradient step (\blacktriangle) and for the final plasma membrane fraction (\blacksquare).

Schüll). Filters were washed once with 2 ml ice-cold 0.1 M LiCl, and were transferred to scintillation vials. The amount of radioactivity was determined with a liquid scintillation counter (Packard Tri-Carb 460 CD; Packard Instruments). Cells were de-energized by preincubation with the protonophore carbamoyl-cyanide-*m*-chloro-phenylhydrazine ($\text{CF}_3\text{-O-Ph}_2\text{C(CN)}_2$, 10 μ M) for 5 min at 25°C.

For uptake studies with hybrid membranes, vesicles were suspended to a final concentration of approximately 1.2 mg protein/ml in 50 mM potassium phosphate (pH 6.5) containing 5 mM MgSO_4 . After a 1-min incubation in the presence of the electron donor system ascorbate (30 mM), $\text{Ph(NMe}_2)_2$ (150 μ M), horse heart cytochrome *c* (7.5 μ M) and L-[U- ^{14}C]-amino acids were added. Samples of 20 μ l were taken at given time intervals, and processed as described above.

Other methods

Protein concentrations were determined in the presence of 0.5% (mass/vol.) SDS using a modified Lowry assay [24]. Bovine serum albumin was used as a standard. The phospholipid content of plasma membranes was determined as described by Rouser et al. [25]. Carbohydrate was assayed using the phenol/sulphuric acid procedure [26]. D-glucose was used as a standard. Total sterols were assayed as described by Rose et al. [27].

RESULTS

Isolation of plasma membranes

Plasma membranes were isolated from two *P. chrysogenum* strains, i.e. Wisconsin 54-1255 and Panlabs P2. These strains differ significantly in morphology and in their capacity to produce penicillin. A final penicillin titre of 25 mM can be reached by the P2 strain, while the Wisconsin 54-1255 strain produces approximately tenfold lower titres. *P. chrysogenum* possesses a thick and rigid cellular wall and therefore only a few homogenization procedures can be used to break whole cells. Fast mechanical disruption of mycelia

with glass beads using a Braun MSK homogenizer appeared to be the most convenient method. Short homogenization times with large glass beads were used to prevent excessive disruption of cell organelles. Within 2 min, more than 95% of the cells were broken. Under these conditions, at least 70% of the mitochondria remained intact as judged from the latency of malate dehydrogenase activity. After homogenization and removal of whole cells and debris, plasma membranes were isolated by Percoll gradient centrifugation and a three-step Nycodenz gradient. Depending on the construction of the Nycodenz gradient, a second fraction of plasma membranes at 1.15–1.17 g/ml was obtained. These plasma membranes are highly permeable to protons and, even after fusion with liposomes containing cytochrome-*c* oxidase, no substantial proton gradient was generated.

To determine the extent of contamination by other membranes, membrane fractions were characterized with the use of biochemical and morphological markers as described by Morrè et al. [28]. Plasma membranes from *P. chrysogenum* contain a vanadate-sensitive P-type ATPase that proved to be a convenient and reliable marker. Like other plant and fungal P-type ATPases [12, 29], the ATPase activity is dependent on magnesium and is stimulated by potassium. Furthermore, the activity is significantly inhibited by vanadate (100 μ M), while azide (<5 mM), nitrate (<50 mM) and oligomycin (<100 μ M) are ineffective. The specific activity of the vanadate-sensitive ATPase increased approximately 25-fold during the isolation procedure (Fig. 1, Table 1). Cytochrome-*c* oxidase, α -D-mannosidase and glucose-6-phosphatase were used as markers for the inner mitochondrial membrane, vacuolar membrane and the endoplasmic reticulum, respectively. α -D-mannosidase is only loosely attached to the vacuolar membrane [30], therefore several control experiments were performed to ensure that the measured activities reflected the actual content of vacuolar membranes in different fractions. All α -D-mannosidase activity could be pelleted by centrifugation at 100000 *g* for 1 h, and the sedimentation of α -D-mannosidase and nitrate-sensitive ATPase activities coincided during several differential centrifugation steps (data not shown). From the marker enzyme activities, it can be con-

Table 1. Marker enzyme activities and protein content of fractions obtained during the isolation of plasma membranes from the *P. chrysogenum* strains P2 and Wisconsin 54-1255. Data are based on the use of approximately 10 g mycelia (dry mass) as the starting material. Fractions were obtained as described in the Experimental Procedures section. The H^+ -ATPase activity is the vanadate (100 μ M)-sensitive activity. Values in parentheses indicate the total activity (%). —, not determined.

Strain	Fraction	Specific activity of				Protein
		H^+ -ATPase	cytochrome- <i>c</i> oxidase	α -D-mannosidase	glucose-6-phosphatase	
		$\mu\text{mol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$				mg
P2	homogenate	0.04 (100)	200 (100)	1.4×10^{-3} (100)	0.10 (100)	4200
	fraction 1	0.04 (49)	410 (92)	2.5×10^{-3} (81)	0.17 (78)	1885
	fraction 2	—	—	—	—	1500
	fraction 3	0.17 (32)	410 (14)	1.8×10^{-3} (9)	0.21 (15)	300
	plasma membranes	0.80 (22)	20 (0.1)	9.0×10^{-3} (0.8)	0.01 (0.1)	46
Wisconsin 54-1255	homogenate	0.08 (100)	70 (100)	0.6×10^{-3} (100)	0.20 (100)	3800
	fraction 1	0.03 (16)	140 (84)	1.1×10^{-3} (80)	0.32 (69)	1650
	fraction 2	—	—	—	—	1325
	fraction 3	0.25 (30)	140 (19)	0.7×10^{-3} (12)	0.44 (21)	360
	plasma membranes	1.90 (46)	10 (0.3)	0.2×10^{-3} (0.5)	0.02 (0.2)	72

cluded that the majority of contaminating membranes is removed by the Percoll gradient step. The specific activity of the plasma-membrane ATPase increased approximately eightfold for both strains by this step. Remaining contaminants were effectively removed by the three-step Nycodenz gradient. For both strains, an approximately similar increase in specific activity of the plasma membrane ATPase was obtained although the yields differed (Table 1).

Inhibitor studies with homogenates of both *P. chrysogenum* strains revealed the presence of three predominant ATPase activities that differed in pH optimum and sensitivity towards inhibitors (Fig. 1A). The activities can be attributed to a vanadate-sensitive P-type ATPase with pH optimum of pH 6.3, a nitrate-sensitive V-type ATPase with an intermediate pH optimum of pH 7.5, and an azide-sensitive F_0F_1 -type ATPase with a pH optimum of approximately pH 9. The purified plasma membranes showed only a high ATPase activity at approximately pH 6 (Fig. 1B), while P2 membranes contained contaminating vacuolar membranes as indicated by a small peak in the ATPase profile at approximately pH 7.5 (Fig. 1B).

Phosphotungstic acid staining was used as a morphological marker for the plasma membranes (Fig. 2). Under appropriate conditions, phosphotungstic acid stains specifically plant and fungal plasma membranes [17]. Whole cells and protoplasts of *P. chrysogenum* showed a distinct staining of the plasma membrane only (Fig. 2A). Morphometric determination of the amount of membranes stained in the final membrane fractions was 90% for the Wisconsin 54-1255 strain (Fig. 2C) and 82% for the P2 strain. The electron microscopical data demonstrate that the final membrane fractions were devoid from other organelles, such as the nucleus or microbody membranes, for which no suitable marker enzyme was available. These data indicate that this isolation procedure allows a significant level of purification of plasma membranes from both strains with high yield.

Properties of *P. chrysogenum* plasma membranes

Some general properties of the *P. chrysogenum* plasma membranes are summarized in Table 2. The density of plasma membranes was assessed by isopycnic sucrose gradi-

ent centrifugation. The broad white plasma membrane band was found at a density of 1.16–1.20 g/ml, and coincided with the protein peak and plasma membrane ATPase activity peak (data not shown).

The plasma membrane from *P. chrysogenum* shows in some aspects a remarkable resemblance with the *Neurospora crassa* plasma membrane. Like the data reported by Bowman et al. [31], a high sterol content and intermediate carbohydrate content (Table 2) were evident.

Fusion of membrane vesicles

For transport studies, plasma membranes were fused with cytochrome-*c* oxidase-containing vesicles by the freeze-thaw method. Freeze fracture images of plasma membranes (Fig. 3B) from the Wisconsin strain revealed a strong difference in particle density between the P-face (cytoplasmic, convex) and the E-face (data not shown). After fusion with cytochrome-*c* oxidase vesicles, this difference was less pronounced and the particle distribution was intermediate between that of cytochrome-*c* oxidase vesicles and plasma membranes (Fig. 3C). After fusion, no membranes with the particle density and distribution of plasma membranes could be observed indicating that all plasma membranes had fused with cytochrome-*c* oxidase vesicles.

Orientation of membrane vesicles

Electron microscopy and Nycodenz gradient centrifugation indicated that most of the isolated plasma membrane vesicles are closed and unilamellar with a diameter of 300–800 nm. The sidedness of the plasma membranes and hybrid membrane vesicles was determined by inhibition of ATPase activity by trypsin. Since trypsin cannot penetrate the membrane, the catalytic domain of the ATPase will not be digested when the cytoplasmic surface is located on the inner face of the membrane. Plasma membranes of the Wisconsin 54-1255 strain are almost completely right-side out (Table 3) as the ATPase was inactivated only when Triton X-100 was present during trypsin digestion. This observation was confirmed by freeze-fracture studies (data not shown). Plasma membrane vesicles obtained from the P2 strain were more

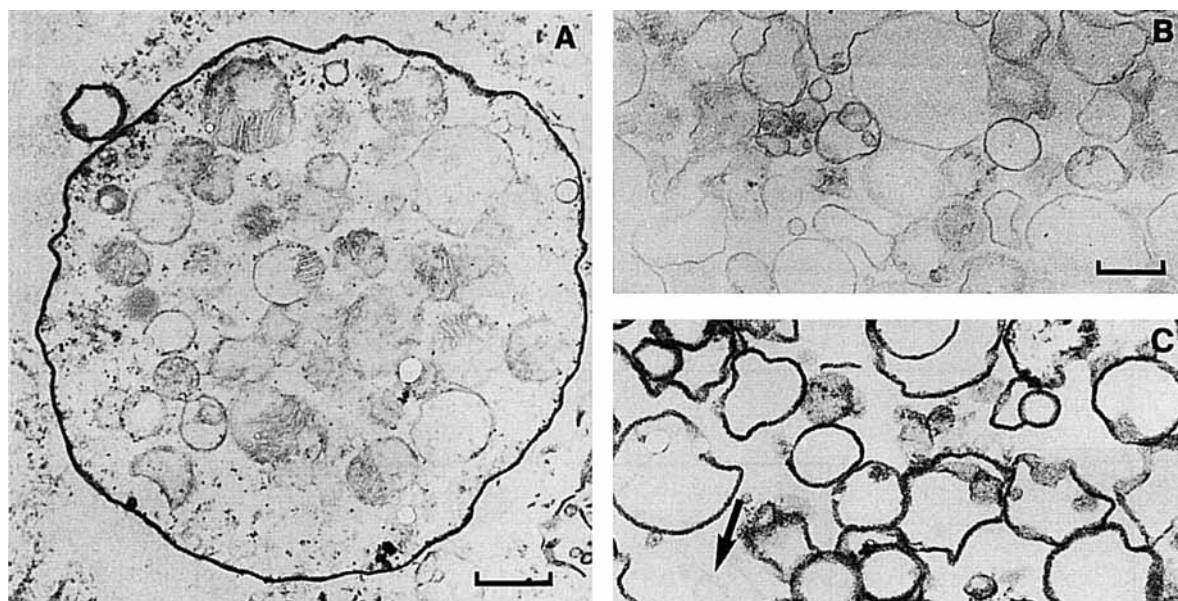


Fig. 2. Phosphotungstic-acid-stained thin sections. (A) Protoplast obtained from the Wisconsin 54-1255 strain stained with phosphotungstic acid. The plasma membrane is heavily stained while none of the intracellular membranes is stained; the bar denotes 0.5 μm . (B, C) Plasma membranes isolated from the Wisconsin 54-1255 strain, etched and stained with phosphotungstic acid (C). A non-stained vesicle is indicated (\leftarrow); the bar denotes 0.25 μm .

Table 2. Characteristics of *P. chrysogenum* plasma membranes. The glucose and protein contents are relative to the amount of phospholipid.

<i>P. chrysogenum</i> strain	Sterol content	Glucose content	Protein content	Density
	mol sterol/(mol sterol + mol phospholipid)	g/g phospholipid		g/ml
P2	20	0.92	0.57	1.19
Wisconsin 54-1255	23	1.12	0.42	1.18

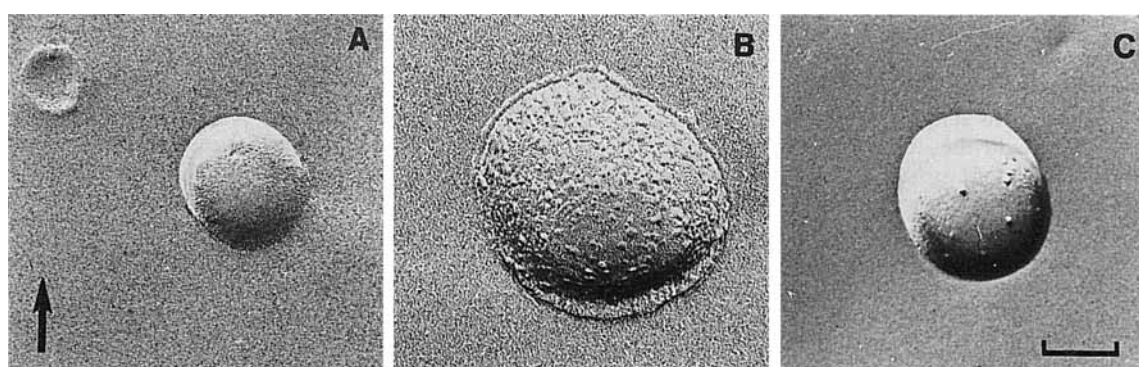


Fig. 3. Freeze-fracture micrographs of a cytochrome oxidase vesicle (A), a plasma membrane vesicle (B) and a hybrid membrane vesicle (C). Plasma membranes were obtained from the Wisconsin 54-1255 strain; hybrid membranes resulted from the fusion of plasma membranes from this strain with cytochrome-*c* oxidase vesicles. The bar denotes 0.1 μm . The direction of shadowing is indicated (\leftarrow).

heterogeneous in orientation, and approximately 50% ATPase activity was accessible from the outside. After fusion of the Wisconsin 54-1255 plasma membranes with liposomes, and subsequent sizing through extrusion, some 'scrambling' of the orientation of the ATPase took place (i.e. approximately 25% of the activity was accessible). The diameter of the fused membranes was 190–240 nm (data not shown).

Amino acid transport

Based on transport studies in mycelia (Fig. 4A and C), the amino acids arginine and valine were used to analyze the transport activity of hybrid membrane vesicles. The uptake of arginine was approximately five times higher in Wisconsin 54-1255 mycelia as compared to the P2 strain. Valine accu-

Table 3. Sensitivity of the plasma membrane ATPase activity towards trypsin. ATPase activity measured without additions was set to 100%. Triton X-100 was added to a final concentration of 0.1% (mass/vol.).

Additions		Relative ATPase activity			
trypsin	Triton X-100	Wisconsin 54-1255		P2	
		plasma membranes	hybrid membranes	plasma membranes	hybrid membranes
		%			
–	–	97 ± 6.5	104 ± 7.9	92 ± 14	98 ± 5.1
+	–	97 ± 2.0	76 ± 5.5	51 ± 3.3	54 ± 4.5
+	+	5 ± 1.4	5 ± 4.6	6 ± 1.2	4 ± 1.6

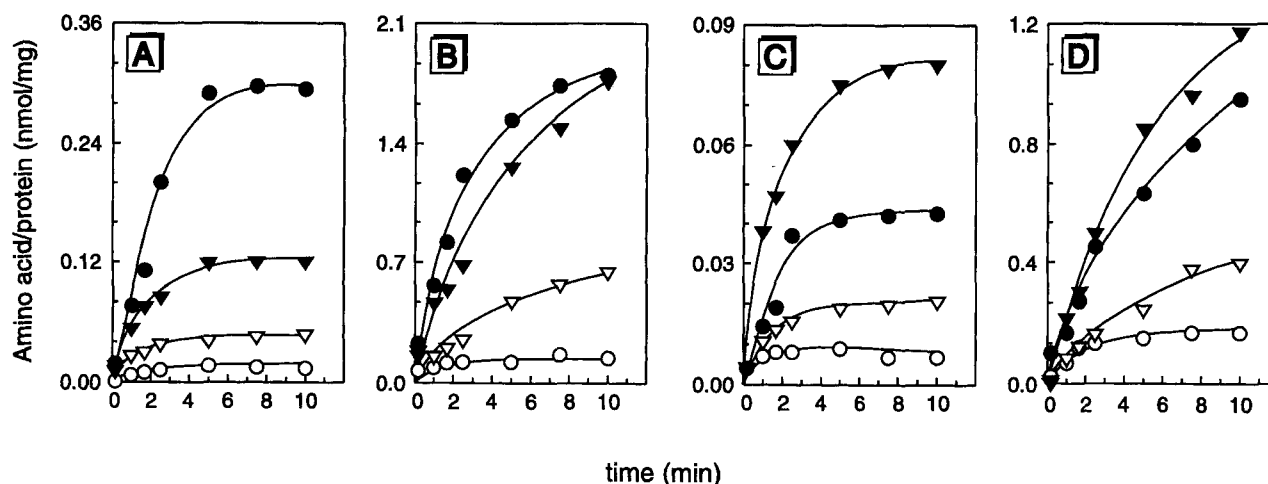


Fig. 4. Uptake of L-arginine and L-valine in mycelia and hybrid membranes. Uptake of arginine (●,○) and valine (▼,▽) in mycelia of the Wisconsin 54-1255 strain (A) or the P2 strain (C). Closed symbols represent uptake under energized conditions, open symbols represent uptake after previous incubation with $\text{CF}_3\text{OPh}_2\text{C}(\text{CN})_2$. The uptake of arginine (●,○) or valine (▼,▽) in hybrid membranes from the Wisconsin 54-1255 strain (B) or the P2 strain (D) are also shown. Closed symbols represent uptake after the addition of ascorbate/ $\text{Ph}(\text{NMe}_2)_2$ /cytochrome *c*, open symbols represent uptake without the addition of redox mediators or after the addition of $\text{CF}_3\text{OPh}_2\text{C}(\text{CN})_2$.

mulation was comparable for both strains. Preincubation of mycelia with the protonophore $\text{CF}_3\text{OPh}_2\text{C}(\text{CN})_2$ completely abolished the uptake of arginine and valine, suggesting that the uptake of these amino acids is Δp dependent.

When incubated with the electron donor system ascorbate, $\text{Ph}(\text{NMe}_2)_2$ and cytochrome *c*, hybrid membranes generated a high Δp , with a transmembrane electrical potential, $\Delta\psi$, of -120 mV and a transmembrane pH gradient, ΔpH , of 60 mV. Hybrid membranes prepared from both strains showed a high level of arginine and valine uptake under these conditions (Fig. 4B and D). Only in the case of Wisconsin 54-1255 membranes did arginine uptake reach a steady-state level within the recorded period. A substantially lower accumulation of valine and arginine was observed when uptake experiments were performed in the presence of $\text{CF}_3\text{OPh}_2\text{C}(\text{CN})_2$ or when experiments were performed in the absence of the electron donors. These results demonstrate that arginine and valine uptake is driven by the Δp or one of its components. The transport activity for both amino acids purified with the plasma membranes (data not shown). Both in whole cells and in hybrid membrane vesicles, the addition of a 100-fold molar excess of unlabeled arginine did not affect the uptake of valine and vice versa, suggesting that distinct transport systems are involved in the uptake of these amino acids.

DISCUSSION

Our primary objective with *P. chrysogenum* is to study specific transport processes that are associated with penicillin production. Therefore, an isolation procedure for pure plasma membranes was developed. Whole cells, instead of protoplasts, were used as starting material for the isolation of plasma membranes because of the ease by which protoplast can be obtained from *P. chrysogenum* differs markedly from strain to strain, and depends strongly on growth conditions and age of the culture. After fusion of the plasma membranes with cytochrome-*c* oxidase vesicles, a Δp can be generated that drives the uptake of different amino acids via transport systems that reside in the fungal plasma membrane. The isolation procedure is applicable to different strains, although different yields are obtained. Cytochemical staining with phosphotungstic acid was used to determine the absolute plasma membrane content of the fractions with the highest purity. This method permits a direct assessment of the purity of an isolated fraction [12].

The plasma membranes derived from the Wisconsin and P2 strains differed significantly in orientation. The cause of this remarkable difference is obscure, but one may speculate that the cytoskeleton is more firmly attached to the plasma membrane of the Wisconsin strain as compared to the P2

strain. The purified plasma membranes showed a density and sterol content that is typical for fungal and mammalian plasma membranes [34]. Moreover, the vanadate sensitive ATPase of the membranes is characteristic of plasma-membrane-associated P-type ATPases. The amino acid uptake studies with the hybrid membranes demonstrate that the system is suitable for the study of active transport processes. The transport activity for arginine and valine purified with the plasma membranes, indicating that this activity is not due to remaining minor contaminants. Since uptake was driven by the Δp (inside negative and alkaline), transport activity cannot be due to vacuolar contaminants as the organelles contain proton/solute antiport systems [30].

In conclusion, this study demonstrates the isolation of *P. chrysogenum* plasma membranes that are active for solute uptake after fusion with cytochrome-c oxidase vesicles. This system is currently being used to characterize the uptake of penicillin precursors and the mechanism of antibiotic excretion.

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